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GRANT NUMBER DAMD17-96-1-6140

TITLE: Substrate Induced Conformational Studies of the Hormone Binding Domain of the Human Estrogen Receptor by Fluorine NMR

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REPORT DATE: July 1997

**DTIC QUALITY INSPECTED 2**

TYPE OF REPORT: Annual

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
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19971210 052

## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	July 1997	Annual (1 Jul 96 - 30 Jun 97)	
4. TITLE AND SUBTITLE Substrate Induced Conformational Studies of the Hormone Binding Domain of the Human Estrogen Receptor by Fluorine NMR		5. FUNDING NUMBERS DAMD17-96-1-6140	
6. AUTHOR(S) Luck, Linda, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Clarkson University Potsdam, New York 13699-5630		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, MD 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Estrogen has been shown to be involved in the progression of breast cancer and the estrogen receptor (ER) has been implicated in reproductive cancers. Our laboratory would like to understand how the structure of the hormone binding domain (HBD) of the ER changes when it is "activated" by estrogens and anti-estrogens. Since the binding of estradiol to its receptor ultimately leads to diverse biological responses to the hormone, we would like to investigate the response of the receptor to a series of ligands and elucidate the molecular basis for their functional differences. This proposal will characterize the critical substrate induced conformational changes in the HBD by incorporating fluorine labels into two recombine constructs and performing fluorine nuclear magnetic resonance (NMR) studies. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor and will compare these changes to those induced by "environmental" estrogens. The purpose of this proposal is to provide and understand the conformational changes in HBD which will shed light on the molecular events of substrate binding, transcriptional activation and the role of environmental estrogens in receptor function.			
14. SUBJECT TERMS Estrogen receptor, breast cancer, NMR, environmental estrogens		15. NUMBER OF PAGES 14	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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Linda A. Luck  
PI - Signature Date

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## INTRODUCTION

The biological importance of estrogen is noted by the number of disease states associated with altered production of estrogen or estrogen like materials. Estrogen has been shown to be involved in the progression of breast cancer and the estrogen receptor (ER) has been implicated in reproductive cancers. One early step in the process of transcriptional activation by estradiol is the production of conformational changes in the ER upon ligand binding. Our laboratory would like to understand how the structure of the hormone binding domain (HBD) of the ER changes when it is "activated" by estrogens and antiestrogens. Since the binding of estradiol to its receptor ultimately leads to diverse biological responses to the hormone, we would like to investigate the response of the receptor to a series of ligands and elucidate the molecular basis for their functional differences. This proposal will characterize the critical substrate induced conformational changes in the HBD by incorporating fluorine labels into two recombinant constructs and performing fluorine nuclear magnetic resonance (NMR) studies. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor and will compare these changes to those induced by "environmental" estrogens. **The purpose of this proposal is to provide an understanding of the conformational changes in HBD which will shed light on the molecular events of substrate binding, transcriptional activation and the role of environmental estrogens in receptor function.**

*Conformational changes in the estrogen receptor.* ER is a member of the steroid receptor superfamily which includes proteins whose functions are to bind small hydrophobic ligands and mediate transcriptional activity within the nucleus.<sup>1,2</sup> These receptor proteins have four distinct actions which modulate biological activity: (1) binding hormone, (2) forming multimeric complexes, (3) binding to sequence specific DNA, known as hormone response elements and (4) modulating transcription. As a consequence of this action DNA synthesis is changed as well as RNA and proteins necessary for cell proliferation. Studies have revealed distinct functional domains in these proteins which include (1) the N-terminal domain which is postulated to be involved in transcriptional enhancement (2) a DNA binding domain, and (3) HBD at the C-terminal end which is responsible for high-affinity binding of steroids. Participation of accessory proteins have been the subject much investigation and have indicated that ER function depends on association with other proteins.<sup>3</sup> In the absence of estrogen, ER is bound in a large molecular complex which includes heat-shock protein 90 (HSP90), HSP70, P59, and other proteins.<sup>4,5</sup> Binding of estrogen to ER releases the monomeric receptor from the complex by inducing a conformational change in the HBD. This conformational change is key to the cascade of events leading to transcription and subsequent protein production.<sup>6</sup> In addition, conformational change is important in dimer formation of ER which allows cooperative ligand binding lowering the ligand concentration range for full action of the protein.

The HBD is of great interest because it contains the regulatory actions of the protein. It is the interaction of the hormone-receptor complex with response elements that can result in induction

or repression of transcription. Chimeric constructs have been made with unrelated proteins such as the *myc* oncogene product which show hormone regulation of the fused gene product. This suggests that the HBD alone is responsible for its own action of ligand binding and conformational change. The interplay between the ligand and the HBD may carefully select how the biologies are manifested.

Many studies have proposed a conformational transition upon binding of estrogen but there is a dearth of information concerning the molecular basis of these changes in the receptor itself. An *in vivo* study from Duke University using a protease digestion assay indicated distinct conformational changes within the ER diverse for antagonists and agonists.<sup>7</sup> Using affinity partitioning in the presence of PEG-palmitate Gorski's laboratory demonstrated a decrease of surface hydrophobicity of ER from rat uterus upon binding estrogen thus indicating conformational change as ligand binds.<sup>8</sup> Characterization of the HBD has been limited in the past for two reasons: difficulty in obtaining large amounts of material and solubility of the protein. Two recent studies have reported expression and purification methods to obtain isolated HBD in high yields.<sup>9,10</sup> The isolated HBD fragment produced in the Greene laboratory was used for mass spectroscopy and ligand binding analysis.<sup>11</sup> Although there has been considerable effort in determining ligand binding and dimerization little has been done to characterize the conformational changes in HBD.

There has been much interest in the steroid hormone estrogen and its interaction with ER since it is a key modulator of differentiation, homeostasis, and development of the female reproductive system.<sup>12</sup> The hormone extends its power beyond reproduction and has been implicated in bone cell biology, and cholesterol and lipid homeostasis in females.<sup>13</sup> A host of disease states including cancer, osteoporosis, and endometriosis have been associated with altered production of estrogen or abnormalities in the way in which the cell responds to estrogen or estrogen-like materials<sup>14,15</sup>

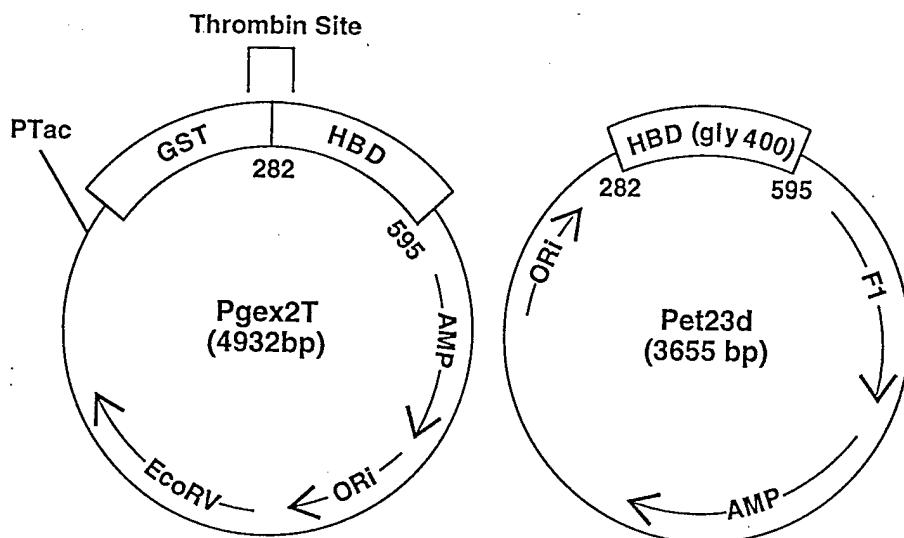
Recent reports have attributed the increase in reproductive abnormalities in wildlife to xenobiotic estrogens which mimic the effects of the naturally occurring estrogens by binding to the estrogen receptor.<sup>16,17,18</sup> Such studies have also hypothesized that environmental agents may be responsible for adverse health consequences in women including disruption of the endocrine system and reproductive cancers.<sup>19</sup> A recent article in *Science* showed that a yeast system containing the human estrogen receptor responded to a combination of environmental toxins in a transactivation assay.<sup>20</sup> This has opened the door to probing the synergistic effects of many pollutants on the hormone system and brought up many questions as to the role of the estrogen receptor and how it binds these agents.<sup>21</sup> Thus we have a need to understand the underlying molecular basis for the function of ER and take a creative approach to examining the HBD in order to provide insight into the mechanism of action of estrogens, antiestrogens and hormone disrupting agents.

**The underlying premise in this proposal is that conformational changes in the ER are crucial to the function of the receptor and its interaction with associated proteins and**

nucleic acids. The initial conformational change upon ligand binding may play the most important role regulating the course of action of the receptor in subsequent transcriptional events. We hypothesize that the initial structural form of the liganded receptor is the pivot point for the future events within the nucleus. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor. We will compare these changes to those induced by "environmental" estrogens.

## BODY

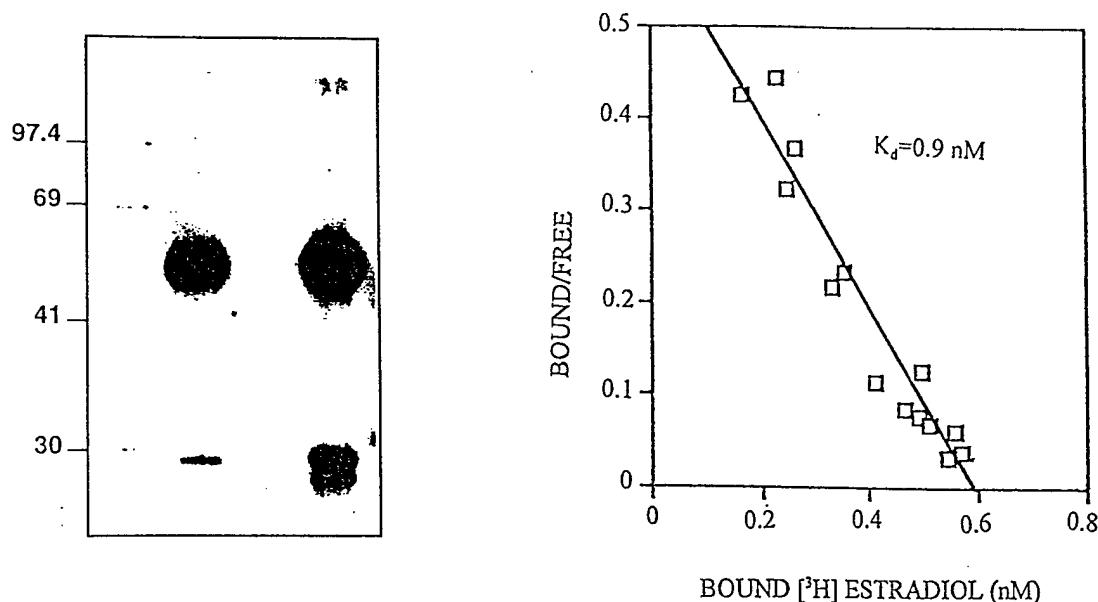
**SYSTEM SET UP** The first goal in this study will be to produce recombinant HBD with fluorine labels for study by NMR. Figure 1 shows the plasmid maps of the two constructs used in this study. The first construct which contains the fusion protein glutathione-S-transferase in tandem with the amino acids 282-595 of the hormone binding domain of the human estrogen receptor (GST-HBD) was made in the Pgex2T vector from Pharmacia. The second construct (GG) was obtained from Geoff Greene's laboratory <sup>11</sup> which contains the residues 282-595 of the HBD in the vector Pet23d from Novagen. Both constructs were transformed into nonauxotroph *E. Coli* cell lines and produce milligram quantities of unlabeled HBD in rich media.



**Figure 1.** GST-HBD and HBD expression vectors. The Pgex2T plasmid provides expression of the amino acids 282-595 of the human estrogen receptor in addition to the fused 27kDa glutathione-S-transferase protein which is used for protein purification. The Pet23d plasmid generates the HBD (282-595) without additional fused amino acids. This plasmid also contains the glycine at position 400. Purification of protein from this plasmid facilitated by an estradiol affinity column.

**GST-HBD PROTEIN.** Our first goal is fluorine label the protein. To accomplish this the GST-HBD construct was transformed into the cell lines W3110, KA197 and AT2470 which are Trp,

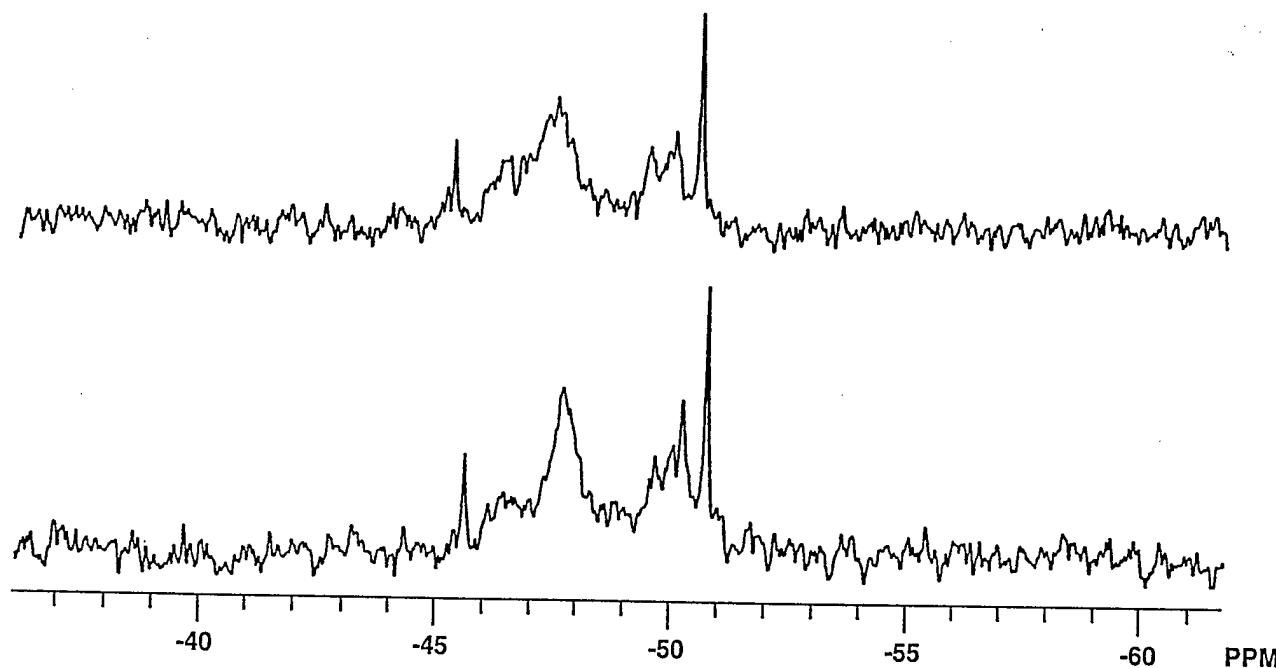
Phe and Tyr auxotroph cell lines, respectively. The GST-HBD plasmid transformed in the W3110 cell line produced protein with 5F-Trp labeling. There are four Trp residues in both the glutathione portion of the protein and the HBD (292, 360, 383, 393). Using a 5:1 ratio of labeled trp to unlabeled trp we were able to get approximately 5 g of pellet per liter of bacteria. Protein purification was accomplished by means of a glutathione sepharose column and elution with 20 mM glutathione. SDS page profile showed the protein with two sets of bands at molecular weight of 55kDa and 30kDa. The thrombin cleaving site between the fusion protein and the HBD self-cleaves during protein purification to a small degree. Western Blot analysis shows positive bands in both areas so the lower bands are consistent with HBD with some slight degradation. (See Figure 2) This proteolysis of the HBD under mild conditions results in a stable protein core of mass 25-30 ka which binds ligand and has been observed in Greene's laboratory<sup>11</sup>. Estradiol binding of the labeled GST-HBD showed a  $K_d$  of 0.9 nM which is the same affinity as the wild type receptor. These data suggest that the fluorine labeled recombinant receptor has not been compromised as far as structural integrity.



**Figure 2.** Protein analysis. (A) Western blot analysis of the GST-HBD purification. The H22 antibody was used in an antibody analysis of the purified GST-HBD. Shown in the first lane are the molecular weight markers. A major band is observed at 58kDa and a minor doublet at 30 ka. An additional band was observed in the higher concentration sample at 116 ka which is consistent with dimerization of the GST-HBD. (B) Binding of [<sup>3</sup>H]-estradiol to labeled GST-HBD. The affinity of 5F-trp labeled GST-HBD was determined by the method of Scatchard. Protein concentrations were determined by Bradford method <sup>23</sup> using IgG as the protein standard.

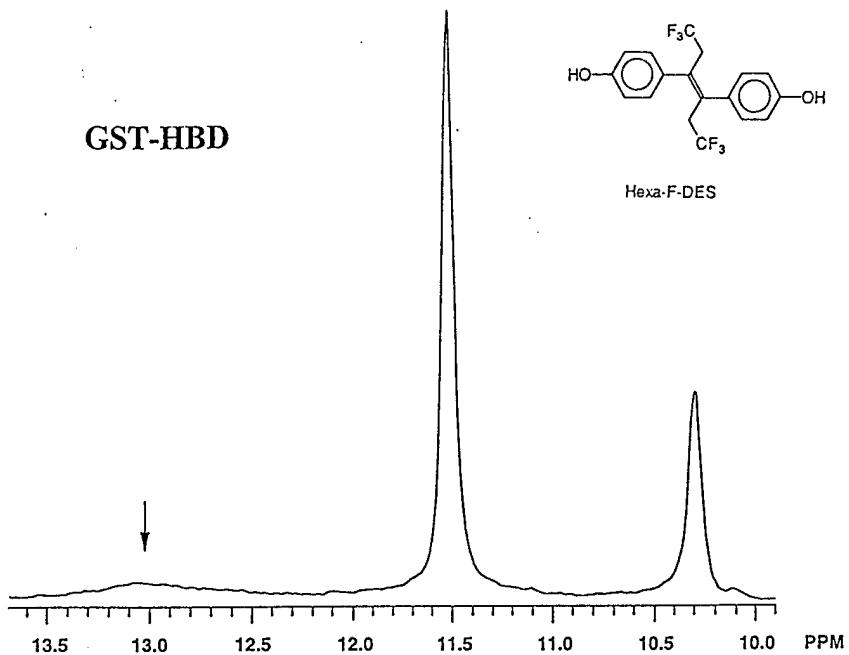
Fluorine NMR analysis of the GST-HBD showed that the 5F-trp labeling was efficient to collect data over a six hour period. Comparison of the protein with and without estradiol shows several

differences in the spectra. The top trace in Figure 3 shows the GST-HBD with an empty site for estradiol. This spectra shows broad lines between -46 ppm and -50.5 ppm. This is consistent with a protein which is undergoing fluctional changes. In contrast the bottom trace shows the protein with the addition of estradiol. This trace shows much sharper lines consistent with a protein in a more rigid conformation. The two sharp lines on either end of the spectrum in both traces may be due to a PMSF adduct formed in the purification or the two tryptophans located on the GST portion of the protein which are fully solvent exposed and have the ability to freely rotate in solution.



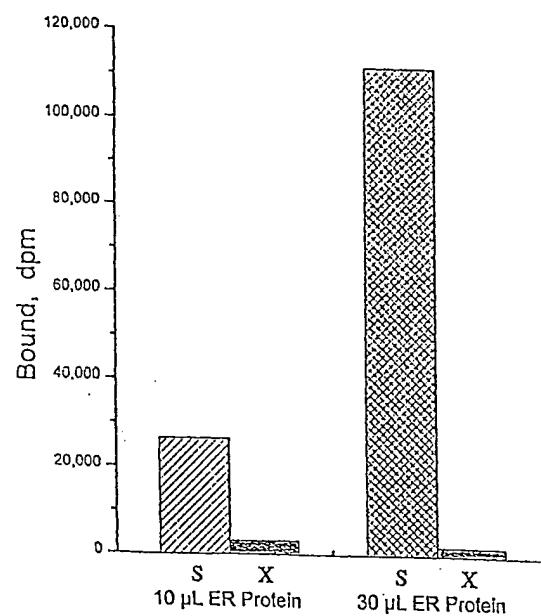
**Figure 3.**  $^{19}\text{F}$  NMR spectra of GST-HBD. Top trace the protein without estradiol. Bottom trace the protein with the addition of estradiol.

To further investigate our recombinant protein we added hexafluorodiethylstilbestrol to an unlabeled sample of GST-HBD. The fluorine NMR of this showed two large peaks at low frequency that correspond to the Z and E isomer of hexafluorodiethylstilbestrol free in solution and a broad peak at 13 ppm which corresponds to the bound form of the ligand. By this experiments we observe that the Z isomer has a much lower intensity and that the HBD has selectively bound to that form. This initial data shows that our fluorine NMR techniques will be useful in sorting out information concerning ligand binding and kinetics.



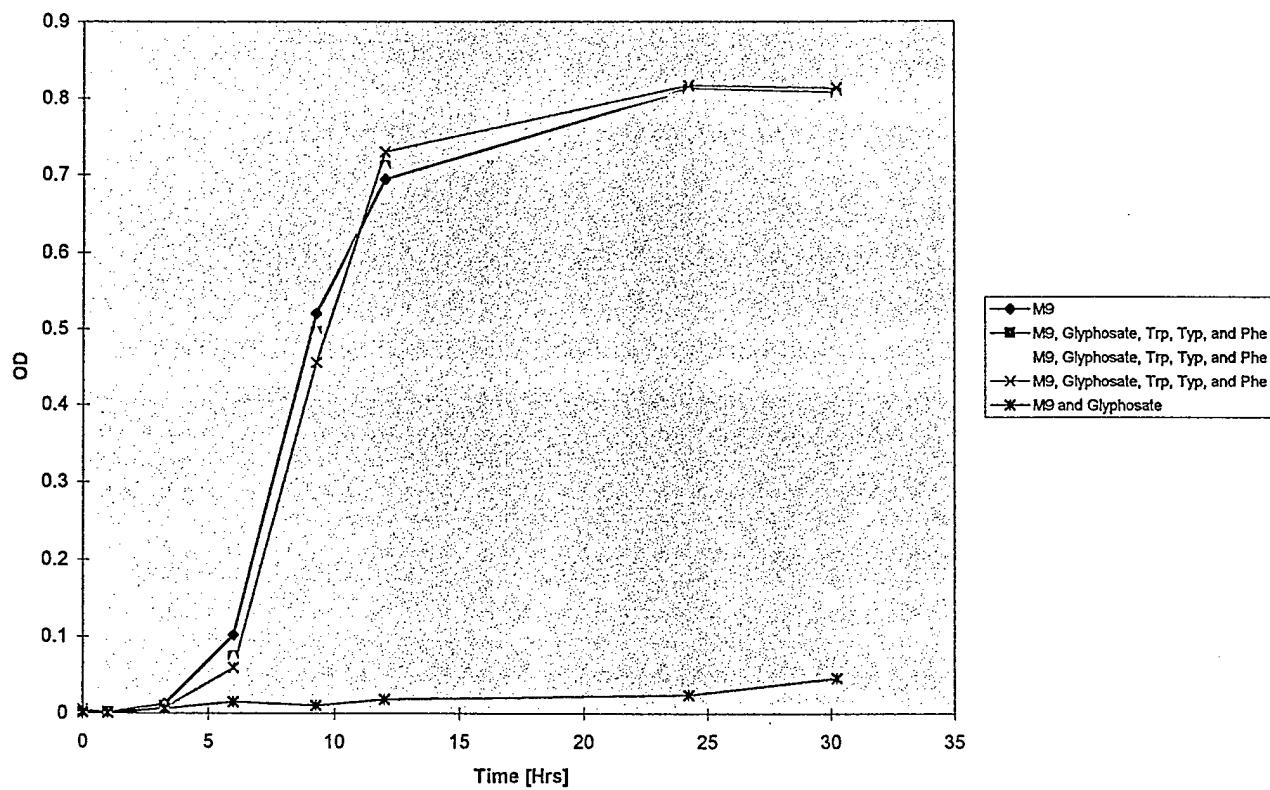
**Figure 4.** Fluorine NMR spectra of hexafluorodiethylstilbestrol and GST-HBD in a 5:1 ratio. The arrow indicates the broad bound peak for the ligand. Spectra are externally standardized to trifluoroacetic acid at 0 ppm.

**HBD PROTEIN.** The protein produced by the GG construct is isolated by the use of a estradiol sepharose column provide by the Greene laboratory. The HBD is eluted with estradiol or other substances that will compete with estradiol for the binding site of HBD. The protein shows a doublet around 33kDa on SDS-Page gels and a positive western blot using AE320 or 311 antibodies from Neomarkers Inc. The HBD is soluble up to 20 mg/ml or .2-.6 mM which is sufficient for all of our NMR studies. Shown in Figure 5 is  $^3\text{H}$ -estradiol binding to HBD and displacement by DES from protein expressed and purified in our laboratory.



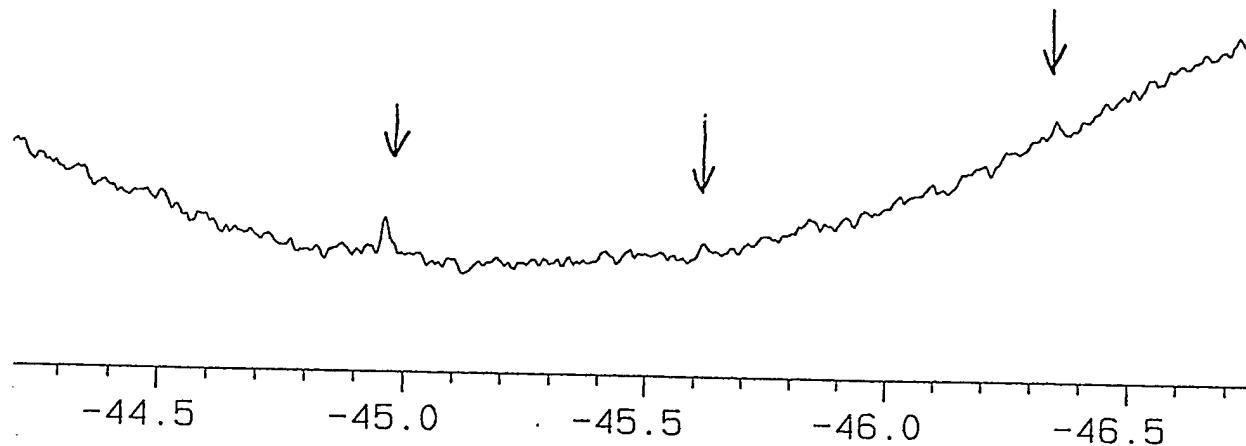
**Figure 5.**  $^3\text{H}$ -Estradiol (3 mM) was incubated in the presence and absence of 200 fold excess of unlabeled DES with 10 or 30  $\mu\text{l}$  of the HBD protein. After incubating for 18 hours at 4°C, 250  $\mu\text{l}$  of HAP(hydroxyapatite) was added to precipitate the  $^3\text{H}$ -estradiol bound protein. After washing 3 times with buffer, the precipitate was pelleted and counted in a scintillation counter.

Our attempts to transform GG into auxotroph cell lines, W3110, AT2471 and KA197 for Trp, Tyr and Phe respectively did not work. We have found that other constructs using pET vectors were also incompatible for transformation with these cell lines. This was a set back for our efforts and we have been forced to search for alternative methods for obtaining fluorinated proteins from non-auxotroph cell lines. Two such methods are (1) addition of large concentrations of fluorinated amino acids to cultures to flood the cells or (2) adding glyphosate which inhibits aromatic amino acid synthesis and adding the appropriate fluorinated amino acid in addition to the unlabeled amino acids necessary for growth.<sup>24</sup> Monsanto has kindly provided us with gram amounts of glyphosate for our experiments so our costs are not prohibitive. We have performed growth curves with minimal media, glyphosate and the various labeled and unlabeled aromatic amino acids to quantitate growth and protein production. See figure 6 for an example of this experiment. Our studies so far have shown that glyphosate with added L-Tyr, L-Phe and 5F-Trp does not inhibit growth of the bacteria and we have been able to get protein production from the plasmid under these conditions. We will investigate a variety of growth conditions to increase the fluorine incorporation and will collaborate with the Ross laboratory and the W. Alton Jones Cell Center Mass Spectroscopy Laboratory to quantitate our results.



**Figure 6.** The growth curve of Pet23d in BL21de3 lys cells. The effect of glyphosate on the growth of cells containing the plasmid for HBD.

By incorporation of glyphosate in the medium along with 5F-trp we have obtained and purified enough protein to collect a fluorine spectrum of HBD. This spectrum shown in figure 7 shows four peaks for the four trp's in HBD. Two peaks are overlapping at -44.9 ppm showing a higher intensity than the two at -45.6 ppm and -46.4 ppm. The concentration of the sample is low and the labeling efficiency is estimated at approximately 50%.



**Figure 7.** The <sup>19</sup>F NMR spectrum of 5F-Trp labeled HBD with estradiol in the binding pocket. Collection time approximately 4 hours with a 1 second time delay between each scan.

We have commenced methods for rigorous assignment of the resonances in this spectrum by site-directed mutagenesis. Oligos for single site replacements of each Trp have been designed. The following is the oligonucleotide for the switch from Trp383 to Phe383 (W383F).

GGATCTCTAGGAAGGCACATTC

## CONCLUSIONS

Using two plasmid constructs for the hormone binding domain of the human estrogen receptor we have found that we can produce protein that is functional in binding estradiol as that found *in vivo*. In addition we have been able to label this protein with 5F-Trp in order to obtain <sup>19</sup>F NMR spectra. The results from the NMR spectra from GST-HBD labeled with 5F-Trp suggest that conformational changes take place in the hormone binding domain of the estrogen receptor when estradiol binds to the pocket. In addition the protein shows a more rigid conformation when the estradiol is present. We have shown that the GST-HBD protein can be used to investigate the binding of fluorinated ligands similar to DES. The HBD protein can be fluorine labeled even without being transformed into a auxotroph cell line by the use of glyphosate in the medium. The spectrum shows four peaks for the four Trp residues for HBD, two are overlapped suggesting that the environments about two tryptophan may be the same in the structure.

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